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Relationship between Histone H3 Lysine 9 Methylation, Transcription Repression, and Heterochromatin Protein 1 Recruitment

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Histone H3 lysine 9 (H3-K9) methylation has been shown to correlate with transcriptional repression and serve as a specific binding site for heterochromatin protein 1 (HP1). In this study, we investigated the relationship between H3-K9 methylation, transcriptional repression, and HP1 recruitment by comparing the effects of tethering two H3-K9-specific histone methyltransferases, SUV39H1 and G9a, to chromatin on transcription and HP1 recruitment. Although both SUV39H1 and G9a induced H3-K9 methylation and repressed transcription, only SUV39H1 was able to recruit HP1 to chromatin. Targeting HP1 to chromatin required not only K9 methylation but also a direct protein-protein interaction between SUV39H1 and HP1. Targeting methyl-K9 or a HP1-interacting region of SUV39H1 alone to chromatin was not sufficient to recruit HP1. We also demonstrate that methyl-K9 can suppress transcription independently of HP1 through a mechanism involving histone deacetylation. In an effort to understand how H3-K9 methylation led to histone deacetylation in both H3 and H4, we found that H3-K9 methylation inhibited histone acetylation by p300 but not its association with chromatin. Collectively, these data indicate that H3-K9 methylation alone can suppress transcription but is insufficient for HP1 recruitment in the context of chromatin exemplifying the importance of chromatin-associated factors in reading the histone code.

In eukaryotic cells, DNA is tightly associated with histones and other factors to form chromatin. The nucleosome is the basic building block of chromatin and consists of approximately 150 bp of DNA coiled around an octamer of histones. The histone octamer contains two copies of each of the core histones, H2A, H2B, H3, and H4. The N-terminal region of each core histone is unstructured when crystallized and therefore is likely to be a highly dynamic structure. These histone tails protrude out from the globular center of the nucleosome where they may interact with nuclear factors. The N-terminal tails are subject to a variety of posttranslational modifications, including phosphorylation, acetylation, methylation, and ubiquitylation. These modifications affect the binding of proteins to the histone tails and thus regulate the nature of the protein complexes that will associate with a region of chromatin. The ability of proteins to specifically associate with certain histone modifications is the basis of the histone code theory (15, 48). According to this theory, specific proteins will associate with histone tails containing certain modifications. These proteins may function to activate or inhibit transcription or serve to maintain a specific chromatin structure.

The best-studied histone modifications are acetylation and methylation. Histone acetylation is generally associated with regions of active transcription. Many transcriptional coactivators contain histone acetyltransferase (HAT) activity, including CBP/p300 (3, 35), the p160 family (46), and P/CAF (63). While arginine methylation of H3 and H4 is associated with transcriptional activation, lysine methylation of histones may have positive or negative effects on transcription, depending on the methylation site(s) (18). Methylation of H3-K9 and H3-K27 is generally associated with repression, whereas methylation of H3-K4, -K36, and -K79 has been implicated in the transcriptional activation process (19, 30, 34, 43, 60). Indeed, the arginine methyltransferases, coactivator-associated arginine methyltransferase 1 (5) and PRMT1 (17), are transcriptional coactivators, while H3-K9 methyltransferases, such as SUV39H1 and G9a (10, 40, 43, 50, 52), are repressors.

Methylation of histone H3 lysine 9 is one of the most highly studied histone modifications. The initial identification of SUV39H1, the human ortholog of *Drosophila Su(var)3-9*, as a lysine 9-specific histone methyltransferase (HMT) and the subsequent finding that heterochromatin protein 1 (HP1) binds specifically to H3 N-terminal tails when methylated on lysine 9 revealed a critical role for lysine 9 methylation in heterochromatin formation and epigenetic control of transcription (1, 4, 20, 27, 29, 32, 40, 43, 52). The connection with HP1 provides a molecular explanation for the general correlation of K9 methylation with transcription silencing or repression. HP1 is a versatile protein that can heterodimerize as well as associate with many other proteins, including histone deacetylases (HDACs), and RNAs (25, 28, 61). Thus, by multiple different interactions, HP1 is believed to create and compact chromatin structure that does not permit transcription. This structure is effectively heterochromatin and is accompanied by lysine 9 methylation and global histone deacetylation.

In addition to SUV39H1 and the highly related SUV39H2, at least two other HMTs, G9a and ESET/SETDB1, have HMT activity toward K9 (44, 49, 62). G9a is likely to be the major euchromatic H3-K9 HMT in mammals, as disruption of the G9a gene resulted in a drastic decrease in H3-K9 methylation mainly in euchromatic regions (50). *G9a*$^{-/-}$ mice are severely growth retarded and die between embryonic day 9.5 and 12.5 due to the inability to repress important developmental genes

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(50). Although an initial study reported that G9a has weak HMT activity toward K27 (49), a more recent study showed that G9a is most likely specific for K9 (38). Furthermore, by using a panel of antibodies specific for mono-, di-, or trimethylated H3-K9 and *G9a*$^{-/-}$ embryonic stem (ES) cells or Suv39h1 and Suv39h2 double-null mouse embryonic fibroblasts, it was recently shown that G9a is mainly responsible for mono- and dimethylation of H3-K9 in euchromatin, whereas Suv39h1 and Suv39h2 direct trimethylation of H3-K9 in pericentric heterochromatin (38, 41).

Although it is known that HP1 binds to both dimethyl- and trimethyl-H3-K9 peptides in vitro (4, 12, 20), their localization patterns do not entirely overlap in vivo. HP1 and methyl-K9 are largely enriched in heterochromatin (14); however, methyl-K9 is also found in euchromatic regions lacking HP1 (23, 41). Since methyl-K9 and HP1 do not completely colocalize, it raises the question as to how HP1 reads the histone code. In addition to its role in heterochromatin, H3-K9 methylation has been reported to be involved in the transcriptional repression of many euchromatic genes (11, 33, 42) and transcriptional repression by thyroid hormone receptor (22). Since methyl-K9 and HP1 do not completely colocalize, whether methyl-K9 can repress transcription via a HP1-independent mechanism remains to be demonstrated. Therefore, the purpose of this study was to determine whether K9 methylation alone is sufficient to recruit HP1 to chromatin and repress transcription.

When single-stranded DNA is introduced into the nucleus of the *Xenopus* oocyte, it rapidly assembles into chromatin via a replication-coupled chromatin assembly pathway, making it an excellent model to study histone modifications, alterations in chromatin structure, and transcription associated with a reporter gene (2). Using this system, we examined whether H3-K9 methylation is sufficient to recruit HP1 and repress transcription. Our results indicate that K9 methylation by itself is not sufficient for recruitment of HP1 to chromatin. In addition, we show that methyl-K9 is sufficient to suppress transcription independent of HP1 recruitment through a mechanism involving histone deacetylation.

MATERIALS AND METHODS

Plasmid constructs and antibodies. The three reporter plasmids, 4xUAS-TRβA-CAT, 4xUAS-TK-CAT, and 4xUAS-AdML-CAT were previously described (21, 22). A full-length cDNA for human G9a was kindly provided by Yoichi Shinka (Kyoto University, Kyoto, Japan). The construct for in vitro synthesis of mRNA encoding Gal4-G9a(SET) was created by PCR amplifying the region C terminal to amino acid 831 and cloning it into a modified pSP64(polyA) vector (Promega, Madison, Wis.) containing an in-frame N-terminal Gal4 DNA-binding domain (DBD) (amino acids 1 to 147). A second Gal4-G9a(SET) construct was created that lacked HMT activity by introducing a point mutation (H1113K) using PCR-directed mutagenesis. His 1113 was chosen for substitution because of its conserved identity with the critical His 324 of SUV39H1. The construct for in vitro synthesis of mRNA encoding Gal4-SUV39H1 and Gal4-SUV39H1 H324K was previously described (22). The constructs for in vitro synthesis of mRNA encoding Gal4 fusions of the N-terminal region (SUVΔC) and the C-terminal HMT domain [SUV(SET)] of SUV39H1 were created by PCR amplification of the N-terminal 118 amino acids or C-terminal 170 amino acids, followed by cloning into the modified pSP64(polyA) vector. Full-length cDNAs for human HP1α, HP1β, and HP1γ were kindly provided by Rafael Herrera (Baylor College of Medicine, Houston, Tex.). These cDNAs were cloned in frame with an N-terminal hemagglutinin (HA) tag by PCR. The 5′ primer was engineered to contain a binding site for the SP6 RNA polymerase, and the 3′ primer was engineered to contain a 15-nucleotide poly(A) tail for in vitro synthesis of mRNA for *Xenopus* oocyte injection.

Acetyl-H3 (K9/K14), acetyl-H4 (K5/K8/K12/K16), acetyl-H3-K9, acetyl-H3-K14, dimethyl H3-K9, dimethyl-H3-K4, dimethyl-H3-K27, and p300 antibodies were purchased from Upstate Biotechnology (Lake Placid, N.Y.). Trimethyl-K9 antibodies were purchased from Novus Biologicals (Littleton, Colo.). Anti-Gal4(DBD) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-HA tag antibody was purchased from Roche (Indianapolis, Ind.).

Microinjection of *Xenopus* oocytes. Preparation and microinjection of mRNA and reporter DNA into stage VI *Xenopus* oocytes was performed as previously described (58). All capped poly(A) mRNAs used for injection were synthesized using a SP6 mESSAGE mACHINE kit (Ambion, Austin, Tex.). Single-stranded DNA of the 4xUAS-TRβA-CAT reporter was prepared as previously described (58). mRNA was injected at a concentration of 100 ng/μl (18.4 nl/oocyte), and reporter DNA was injected at a concentration of 50 ng/μl (18.4 nl/oocyte) according to the experimental scheme described for each experiment.

Western blotting. Western blotting was performed to confirm that injected mRNAs were efficiently translated to correctly sized proteins. *Xenopus* oocytes (5 to 10 oocytes) were homogenized in 100 mM Tris–10 mM EDTA (pH 8.0) (10 μl/oocyte) and centrifuged to remove insoluble material. An equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye was added to each sample and either used immediately for Western blot analysis or stored at −20°C. Western blot analyses were performed as previously described (47) using 20 μl of each sample per well (the equivalent of the protein present in a single oocyte). Primary antibodies were diluted 1:1,000, except for anti-Gal4(DBD) and anti-HA tag antibodies, which were diluted 1:5,000.

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (21). Briefly, oocytes (10 to 20/group) were treated with 1% formaldehyde in MBSH buffer for 10 min at room temperature. The oocytes were then washed twice with high-salt modified Barth's solution [MBSH; 10 mM HEPES, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$] and incubated in 100 mM Tris (pH 9.4)–10 mM dithiothreitol (DTT) for 15 min at 30°C. Next, oocytes were rinsed and homogenized in 800 μl of homogenization buffer (20 mM Tris [pH 7.6], 60 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The extract was sonicated with 50 pulses from a Branson Sonifier 250 at 40% duty cycle and 40% output to break chromatin into fragments with an average length of 500 bp. The sonicated extract was diluted with ChIP I buffer (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.4 mM PMSF) to a final volume of 1.4 ml and centrifuged to remove insoluble material. The supernatant was incubated with Sepharose 4B (Amersham Biosciences) for 1 h at 4°C to remove molecules that nonspecifically bind to the Sepharose beads. After the incubation, the beads were removed by centrifugation, and the supernatant was used for IP. Twenty microliters of supernatant was stored at −20°C and later used as input for PCR. Sonicated chromatin solution (50 to 100 μl) was used in overnight IPs with 1 μg of antibody, 5 μl of protein A/G+ agarose (Santa Cruz Biotechnology) and ChIP I buffer to a final volume of 150 μl. The following day, the beads were washed once with 400 μl of ChIP I buffer, ChIP II buffer (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES [pH 7.5], 500 mM NaCl, 1 mM DTT, 0.4 mM PMSF), ChIP III buffer (0.25 mM LiCl, 0.5% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8.0], 1 mM DTT, 0.4 mM PMSF), and TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) in a cold room. After the last wash buffer was aspirated, 100 μl of elution buffer (0.5% SDS, 0.1 M $NaHCO_3$) plus 5 μg of proteinase K (Roche) was added to the beads and the input samples and incubated overnight at 65°C to reverse formaldehyde cross-links and degrade protein. The following day, the samples were extracted first with phenol-chloroform and then with chloroform. The samples were then ethanol precipitated with 5 μg of glycogen, washed with 70% ethanol, and resuspended in 20 μl of water (40 μl for input samples). Four microliters of each sample was used for PCR.

Standard PCR was performed in 20-μl volumes with the inclusion of 1 μCi of [^{32}P]dCTP. The products were visualized by autoradiography. PCR primers amplified a 100-bp region in the promoter of the 4xUAS-TRβA-CAT reporter and have been previously described (22). Twenty cycles of PCR were performed, with 1 cycle consisting of 45 s at 94°C, 45 s at 63°C, and 45 s at 72°C. All experiments involving ChIP analyses were repeated a minimum of three times for statistical analyses as described below. A representative blot from multiple experiments is shown in each figure.

Primer extension. Primer extension was used to analyze the quantity of RNA transcripts produced form reporter genes in *Xenopus* oocytes. The procedure used for primer extension has been previously described (58). The *Xenopus* oocyte storage histone H4 mRNA was used as an internal control for all primer extension assays (57).

GST pull-down assays. One microgram of glutathione *S*-transferase (GST) or GST-HP1α fusion protein immobilized on glutathione-Sepharose was incubated with 5 μl of the in vitro translation reaction mixture and 200 μl of binding buffer (20 mM HEPES [pH 7.5], 150 mM KCl, 10% glycerol, 0.1% NP-40, 1 mM PMSF, 1 mM DTT) for 2 h at 4°C. The beads were washed five times for 10 min each time at 4°C with binding buffer containing 400 mM NaCl. Bound proteins were eluted with 10 μl of 2× SDS-PAGE loading buffer and separated by SDS-PAGE. Gels were stained with Coomassie blue and destained with water before incubating with Amplify reagent (Amersham Biosciences), drying, and autoradiography.

Coimmunoprecipitation assays. Twenty-five *Xenopus laevis* oocytes were injected with mRNA encoding HA-tagged HP1α (HA-HP1α) and the appropriate Gal4 fusion construct as indicated in the figures. After the oocytes were incubated overnight, they were homogenized in 125 μl of IP buffer (150 mM NaCl, 20 mM Tris [pH 7.6], 1 mM EDTA, 0.2 mM PMSF, 10% glycerol, and aprotinin, pepstatin, and leupeptin [1 μg/ml]) and centrifuged at 4°C to remove insoluble material. The supernatant was collected and used for IP. Each IP reaction mixture was incubated overnight and consisted of 40 μl of extract, 160 μl of IP buffer with 0.5% NP-40, 5 μl of protein A/G Plus Sepharose (Santa Cruz Biotechnology) and 2 μl of either anti-HA or anti-Gal4(DBD) antibody. Five washes were performed for 10 min each time at 4°C with IP buffer plus 0.5% NP-40. The proteins were then denatured and dissociated from the beads by boiling in SDS loading dye before Western blot analysis by SDS-PAGE (12% polyacrylamide). Western blotting was performed as described above using either anti-HA or anti-Gal4(DBD) antibody. Twenty percent of the input (8 μl) for each IP was included on each Western blot.

Statistical analyses. Integrated optical density measurements were obtained from ChIP autoradiographs by scanning densitometry using National Institutes of Health ImageJ v1.33j software and subjected to least-squares analysis of variance (LS-ANOVA) using the general linear model procedures of the Statistical Analysis System v8.1 for Windows (SAS Institute, Inc., Cary, N.C.). Data were analyzed using the chromatin input PCR as a covariate in LS-ANOVA. LS means and standard errors were derived from this analysis. Preplanned orthogonal contrasts were used to test for effects of treatment [e.g., no mRNA injected (control) (Cx) versus G9a(SET), Cx versus SUV39H1, and G9a(SET) versus SUV39H1]. *P* values of ≤0.05 were considered statistically significant. The values reported beneath the ChIP blots in each figure are the fold differences between the LS mean for each group and the control group. Values that are significantly different for the treatment groups are indicated by different superscript letters. The LS means and standard errors and *P* values obtained from these tests are available upon request.

RESULTS

Lysine 9 methylation alone is insufficient for recruitment of HP1 to chromatin. To study the effect of H3-K9 methylation on transcriptional repression and HP1 binding, we created two fusion protein expression constructs by fusing the DBD of Gal4 to either the HMT domain of G9a [Gal-G9a(SET)] or the full-length SUV39H1 (Gal-SUV39H1). Since it is thought that K9 methylation represses transcription by providing a binding site for HP1, we first wished to determine whether K9 methylation alone was sufficient to recruit HP1 to chromatin. In order to test the binding of HP1 to methyl-K9 in vivo, we also prepared constructs in which a HA tag was added to the N terminus of human HP1α, HP1β, or HP1γ. Capped poly(A) mRNA was prepared from these templates by in vitro transcription and injected into *Xenopus* oocytes. After overnight incubation, the expression of protein from injected mRNA was analyzed by Western blotting. As shown in Fig. 1B, the mRNAs were efficiently translated to proteins of the expected sizes.

Next, we tested whether tethering G9a(SET) or SUV39H1 to chromatin would result in H3-K9 methylation. For this purpose, groups of *Xenopus* oocytes were injected with or without mRNA encoding Gal-G9a(SET) or Gal-SUV39H1. Three hours later, a single-stranded DNA reporter (4xUAS-TRβA-CAT) containing four Gal4-binding sites was injected into the oocyte nuclei to assemble the reporter DNA into chromatin (Fig. 1A). After the oocytes were incubated overnight, they were processed for ChIP assay to determine the status of histone modifications over the promoter region. As shown in Fig. 1C, both Gal-G9a(SET) and Gal-SUV39H1 bound the reporter DNA as determined by ChIP assay using anti-Gal4(DBD) antibody. Importantly, expression of either Gal-G9a(SET) or Gal-SUV39H1 led to increased levels of H3-K9 methylation and decreased levels of acetylated K9 [Cx versus G9a(SET), $P \leq 0.02$; Cx versus SUV39H1, $P \leq 0.01$]. In full agreement with a previous report showing that G9a exhibits 20-fold-more HMT activity toward lysine 9 than SUV39H1 (49), a greater HMT activity was observed for Gal-G9a(SET) as determined by ChIP using anti-dimethyl K9 antibody [G9a(SET) versus SUV39H1, $P < 0.0001$] (Fig. 1C). The level of detectable acetyl-K9 was reduced by both G9a(SET) and SUV39H1; however, SUV39H1 did so to a greater extent [Cx versus G9a(SET), $P = 0.02$; Cx versus SUV39H1, $P = 0.001$; G9a(SET) versus SUV39H1, $P = 0.01$] (Fig. 1C). In the original report, G9a was shown to weakly methylate lysine 27 of H3 (49); however, we did not detect any K27 methylation by Gal-G9a(SET) in our ChIP assay. The failure to detect K27 methylation is probably not due to a problem with the antibody used, because the same antibody detected K27 methylation in other experiments (data not shown). Rather, we believe there is little to no K27 methylation activity for G9a. In support of our result, a recent comparison of K27 methylation states in wild-type and *G9a*-null ES cells indicated that G9a may not regulate K27 methylation at all (38).

Having established that tethering both G9a(SET) and SUV39H1 to chromatin results in increased H3-K9 methylation, next we tested whether H3-K9 methylation would be sufficient to recruit HP1 to chromatin. Our initial attempts to perform ChIP assays using antibodies against mammalian HP1 isoforms failed to unambiguously detect the recruitment of endogenous *Xenopus* HP1 proteins, possibly because the antibodies used failed to recognize the *Xenopus* counterparts, or there may be a low level of endogenous HP1 protein in the *Xenopus* oocyte. To circumvent this problem, we expressed HA-tagged human HP1α, HP1β, and HP1γ in the oocytes and performed ChIP experiments using antibodies against the HA tag. As illustrated in Fig. 1D, Gal-SUV39H1 was able to recruit all HP1 isoforms to chromatin; however, Gal-G9a(SET) was not. Together with data in Fig. 1C, these results indicate that lysine 9 methylation alone is insufficient to recruit HP1 to chromatin and suggest that other factors must also be involved in HP1 recruitment.

Lysine 9 methylation can repress transcription independently of HP1 recruitment. On the basis of the fact that the chromo domain (CD) of HP1 binds specifically to H3 N-terminal tail peptides containing methyl-K9 in vitro (4, 20), it is thought that K9 methylation silences gene expression through the recruitment of HP1, which forms a compact chromatin structure that does not permit transcription. However, the results in Fig. 1 indicate that HP1 was not recruited to chromatin after lysine 9 methylation by Gal-G9a(SET), prompting us to test whether K9 methylation could suppress transcription independently of HP1 recruitment. To test this hypothesis, oocytes were injected with mRNA encoding either wild-type Gal-G9a(SET) or Gal-G9a(SET) H1113K, a point mutant

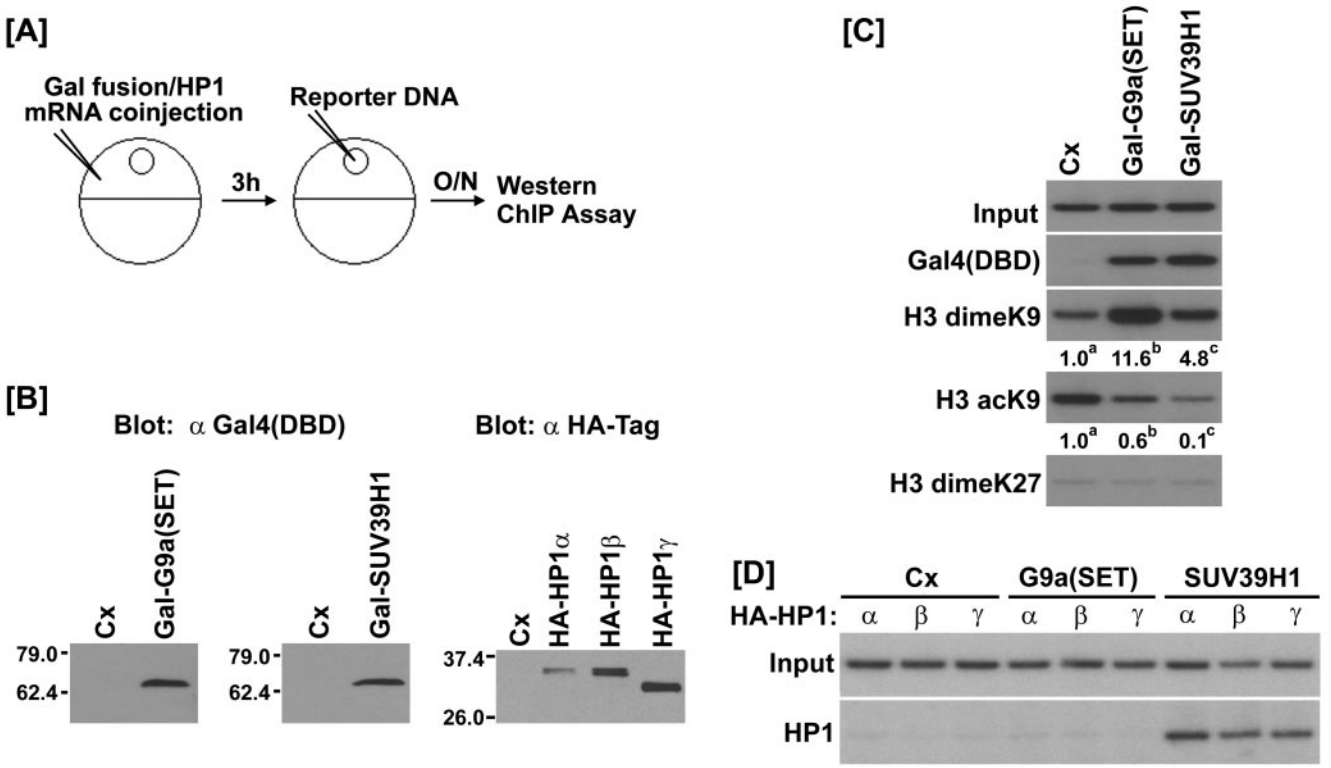

FIG. 1. Lysine 9 methylation alone is insufficient for recruitment of HP1 to chromatin. (A) Experimental design. mRNAs encoding Gal4 fusion proteins [Gal-G9a(SET) and Gal-SUV39H1] and HA-HP1 isoforms were injected into the oocyte cytoplasm. The single-stranded reporter DNA was 4xUAS-TRβA-CAT. The injected oocytes were incubated overnight (O/N) and processed for Western blot analysis and ChIP assay. (B) Western blots showing expression of the Gal4-G9a and Gal4-SUV39H1 fusion proteins and the three HA-tagged HP1 isoforms in *Xenopus* oocyte extracts. The positions of molecular mass markers (in kilodaltons) are shown to the left of the blots. Cx, no mRNA injection; α Gal4(DBD), anti-Gal4(DBD) antibody; α HA-Tag, anti-HA tag antibody. (C) ChIP assays to study modifications to histones associated with the promoter of the reporter DNA. Antibodies against H3 dimethyl-K9 (H3 dimeK9) and H3 dimethyl-K27 (H3 dimeK27) were used in this experiment. ChIP using a Gal4(DBD)-specific antibody was included to show that both fusion proteins bound the reporter. Both Gal-G9a(SET) and Gal-SUV39H1 induced methylation of H3-K9 and decreased H3-K9 acetylation (H3 acK9). The results of these and replicate experiments were analyzed by LS-ANOVA and subject to preplanned orthogonal contrasts. The fold difference between the LS mean for each group and the control group (Cx) is reported below the appropriate lane. Values that are significantly different ($P \leq 0.05$) are indicated by different superscript letters. (D) SUV39H1, but not G9a(SET), was able to recruit all HP1 isoforms to chromatin as determined by ChIP assay using anti-HA tag antibody.

which should render the HMT inactive. Western blot analysis showed that both Gal-G9a(SET) and Gal-G9a(SET) H1113K were expressed at similar levels after overnight incubation (Fig. 2B). To ensure that the H1113K mutation indeed inactivates G9a HMT activity, we tested its ability to target H3-K9 methylation to a chromatinized 4xUAS-TRβA-CAT reporter following the experimental scheme shown in Fig. 2A. Indeed, although both Gal4 fusion proteins bound the reporter DNA as revealed by ChIP assay using Gal4(DBD) antibody, only the wild-type G9a(SET) was able to methylate lysine 9 (Fig. 2C). Again, Gal-G9a(SET) was unable to methylate H3-K27 to a detectable level. H3-K4 methylation was detected in the control group and was unaffected by either Gal-G9a(SET) construct. These data demonstrate that the G9a(SET) H1113K mutant is indeed inactive in H3-K9 HMT activity.

Next we tested the ability of the wild-type and mutant G9a(SET) to repress transcription. The primer extension results in Fig. 2D (top blot) showed that expression of the wild-type Gal-G9a(SET), but not the mutant, repressed transcription from the 4xUAS-TRβA-CAT reporter in a dose-dependent manner. This HMT-dependent repression is not promoter specific, as repression was also observed when two different reporters, one driven by adenovirus major late promoter (4xUAS-AdML-CAT) and one driven by human thymidine kinase promoter (4xUAS-TK-CAT), were tested (Fig. 2D, bottom blot). Since Gal-G9a(SET) repressed transcription but was unable to recruit HP1 (Fig. 1) and since the inactive-HMT mutant failed to repress transcription, these data indicate that lysine 9 methylation is sufficient to suppress transcription independently of HP1 recruitment.

Transcriptional repression by lysine 9 methylation involves histone deacetylation. Since lysine 9 methylation can suppress transcription without HP1 recruitment, we wished to understand the mechanism for this repression. Given the potential functional interplay among different histone modifications, we performed ChIP assays to assess the effect of targeting Gal-G9a(SET) to chromatin on various histone modifications (Fig. 3A). Again, *Xenopus* oocytes were injected with mRNA encoding either wild-type or H1113K Gal-G9a(SET) and the 4xUAS-TRβA-CAT reporter DNA and the effects on histone modifications were determined by ChIP assays. Both Gal-G9a(SET) and Gal-G9a(SET) H1113K proteins bound the

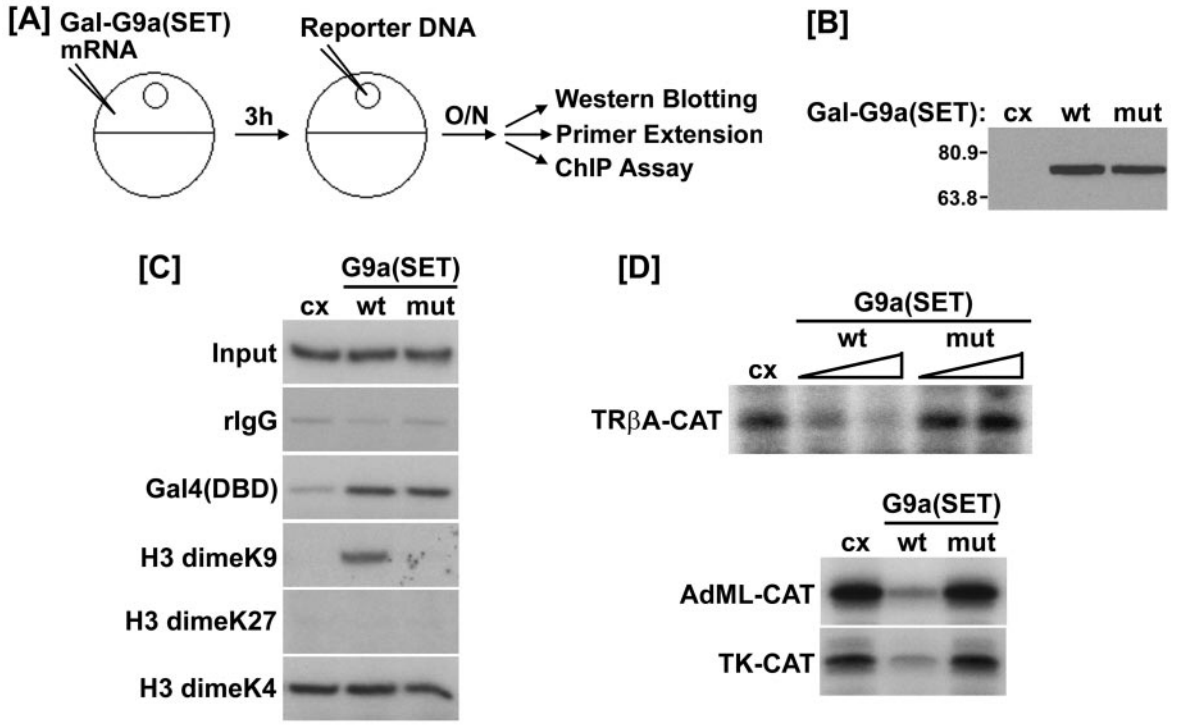

FIG. 2. G9a(SET)-induced H3-K9 methylation is sufficient for transcriptional repression in the absence of HP1 recruitment. (A) Experimental design. The groups of oocytes were injected with the indicated mRNA or DNA, incubated overnight (O/N), and processed by three methods: Western blot analysis to measure protein expression, primer extension analysis to measure transcription, and ChIP assay to study histone modifications. The reporter DNA was 4xUAS-TK-CAT, 4xUAS-AdML-CAT, or 4xUAS-TRβA-CAT. (B) Expression of wild-type (wt) and mutant (mut) H1113K Gal-G9a(SET) proteins in *Xenopus* oocytes as revealed by Western blot analysis using a Gal4(DBD)-specific antibody. (C) ChIP assays showing that both G9a(SET) constructs bound the reporter but that only the wild type was able to methylate K9. Antibodies against H3 dimethyl-K9, -K4, and -K27 were used as indicated (H3 dimeK9, H3 dimethyl-K9). rIgG, normal rabbit IgG. cx, no mRNA injection. (D) The wild-type protein, but not mutant Gal-G9a(SET), suppressed transcription of all three reporter genes as determined by primer extension analysis. For the TRβA-CAT reporter, oocytes were divided into five groups. The first group received no mRNA (cx), and the other groups received one of two concentrations (undiluted or 1:3 dilution) of wild-type (wt) Gal-G9a(SET) or mutant (mut) H1113K mRNA. For the AdML- and TK-CAT reporters, mRNA was injected at a single concentration (undiluted). All the experiments in this figure were repeated three times to ensure reproducibility.

reporter gene equally well as determined by ChIP assays using anti-Gal4(DBD) antibodies. Consistent with the data in Fig. 2C, the wild-type Gal-G9a(SET) methylated lysine 9 (Cx versus $G9a^{wt}$, $P < 0.001$) and reduced H3-K9 acetylation (Cx versus $G9a^{wt}$, $P = 0.009$) whereas the mutant Gal-G9a(SET) did not (Cx versus $G9a^{mut}$, $P = 0.78$ and 0.49). Surprisingly, the effect on acetylation was not restricted to H3-K9, as a significant decrease in acetylation of H3-K14 and even of histone H4 was detected (Cx versus $G9a^{wt}$, $P = 0.009$ and 0.001). These results reveal an unexpected extensive effect of targeting G9a(SET) to chromatin on histone acetylation. In addition, these results provide a potential mechanism for the HP1-independent repression function of K9 methylation.

One potential explanation for the observation that both H3 and H4 were deacetylated upon tethering G9a(SET) to chromatin is that the G9a SET domain interacts with and recruits one or more HDAC activities to chromatin. However, the result that the G9a(SET) H1113K mutant failed to induce K9 methylation and histone deacetylation strongly argues against such a possibility, unless the H1113K mutation also simultaneously abolished its hypothetic interaction with HDAC. Nevertheless, we performed ChIP experiments to determine whether Gal-G9a(SET) could recruit endogenous *Xenopus* Rpd3, Flag-HDAC1, or Flag-HDAC3 to chromatin; however, we were unable to detect recruitment of any of these HDACs (data not shown). Furthermore, in an attempt to pull down HDAC activity from HeLa nuclear extract or *Xenopus* oocyte extract using recombinant GST-G9a(SET), we failed to detect HDAC activity beyond the control GST protein (data not shown). Therefore, we conclude that the observed effect on histone acetylation by tethering G9a(SET) to chromatin is unlikely a result of direct recruitment of HDACs by the G9a(SET) domain but rather an indirect result of H3-K9 methylation. We suggest that K9 methylation could inhibit histone acetylation by preventing acetylation by HATs or by enhancing deacetylation by HDACs or both (see Discussion).

To determine whether repression by lysine 9 methylation is indeed dependent on histone deacetylation, we tested whether the addition of a HDAC inhibitor, trichostatin A (TSA), would block repression by Gal-G9a(SET). *Xenopus* oocytes were injected with mRNA encoding Gal-G9a(SET) or left alone and injected with either the 4xUAS-TK-CAT or 4xUAS-TRβA-CAT reporter. The oocytes were then incubated overnight in the presence or absence of TSA. The primer extension analysis

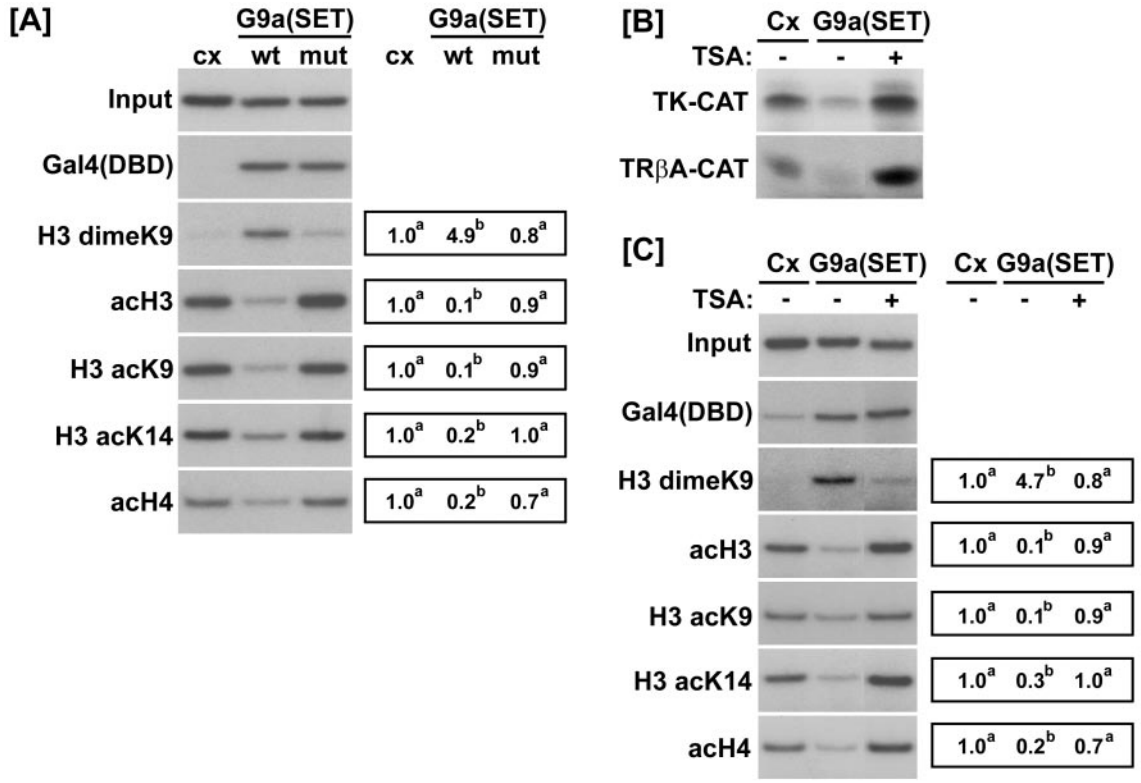

FIG. 3. Transcriptional repression by lysine 9 methylation involves histone deacetylation. (A) ChIP assays comparing the effect of K9 methylation on acetylation of histones H3 (acH3) and H4 (acH4). Groups of oocytes were not injected with mRNA (cx) or were injected with wild-type (wt) Gal-G9a(SET) or mutant (mut) (H1113K) Gal-G9a(SET) mRNA as indicated and the single-stranded 4xUAS-TRβA-CAT reporter. After overnight incubation, ChIP assays were performed using the indicated antibodies. Fold differences between the LS mean for each group and the control group (cx) are reported to the right of the blots. H3 dimeK9, H3 dimethyl-K9; H3 acK9, H3 acetyl-H9. (B) Primer extension assays to determine the role of histone deacetylation on methyl-K9-induced transcriptional repression. The HDAC inhibitor TSA (1.65 μM) was added (+) immediately after injection of reporter DNA. RNA was prepared the following day. Note that TSA blocked repression by Gal-G9a(SET). (C) Effect of inhibiting deacetylase activity with TSA on the histone modifications induced by Gal-G9a(SET). Oocytes were injected as described above for panel B, and ChIP analyses were performed using groups of oocytes injected with the 4xUAS-TRβA-CAT reporter DNA. Note that H3-K9 methylation was almost completely blocked by TSA treatment and that TSA induced histone acetylation. Fold differences between the LS mean for each group and the control group (Cx) are reported to the right of the blots.

showed that TSA treatment completely blocked the repression induced by Gal-G9a(SET) (Fig. 3B). These data indicate that histone deacetylation is essential for repression by K9 methylation.

We also performed ChIP assays using extracts from oocytes injected with the 4xUAS-TRβA-CAT reporter to determine what effect TSA had on the histone modifications we had previously examined. Even in the presence of a functional Gal-G9a(SET) HMT, TSA treatment increased the level of acetylation of both histone H3 and H4 and reduced the level of K9 methylation (G9a versus G9a+TSA, $P < 0.01$) (Fig. 3C). The blockage of K9 methylation in the presence of TSA is probably not due to the inhibition of HMT activity by TSA, because TSA did not affect the HMT activity present in HeLa nuclear extracts and *Xenopus* oocyte extracts in vitro (data not shown). Also, there is no report that G9a or any other HMT can be inhibited directly by TSA. Rather, this result is consistent with the idea that methylation on K9 first requires deacetylation; blocking histone deacetylation by TSA prevents K9 methylation by G9a.

Lysine 9 methylation inhibits histone acetylation by p300 but does not affect its association with chromatin. Most, if not all, HATs contain a bromo domain that binds acetylated lysine residues. Thus, H3-K9 methylation could promote histone deacetylation by affecting the binding of HATs to chromatin and consequently inhibiting histone acetylation. To address this question, we sought to determine whether H3-K9 methylation inhibits the chromatin association and/or activity of the p300 acetyltransferase. Western blots for Gal4-DBD or p300 show that the Gal-G9a(SET) and p300 proteins were expressed in the proper groups (Fig. 4A). ChIP assays were performed to analyze the association of p300 with chromatin and determine the effects on histone modifications (Fig. 4B). Overexpression of p300 increased the amount of p300 associated with the chromatin assembled reporter (Cx versus p300, $P = 0.01$). Interestingly, the H3-K9 methylation induced by Gal-G9a(SET) did not inhibit the association of p300 with chromatin (p300 versus p300+G9a, $P = 0.19$); however, it did substantially inhibit p300-induced H3 acetylation. In this experiment, overexpression of p300 did not significantly increase

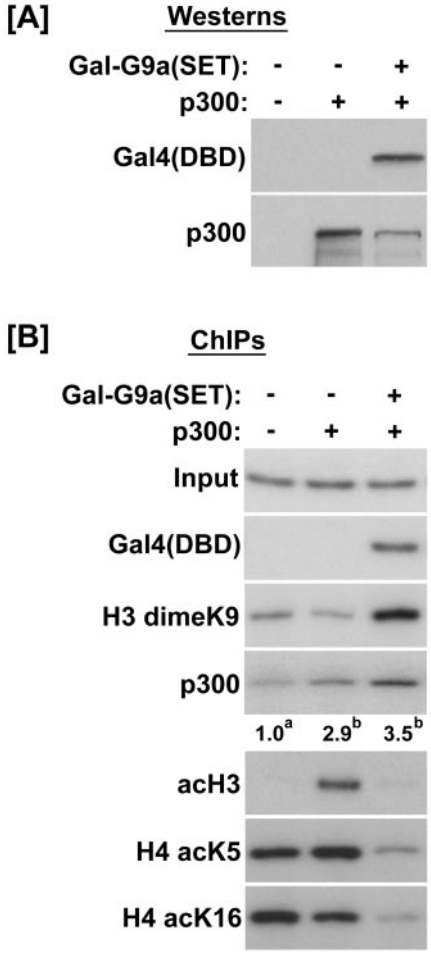

FIG. 4. (A) Western blots showing expression of p300 and Gal-G9a(SET) proteins in *Xenopus* oocyte extracts. (B) ChIP assays to determine the effect of K9 methylation on the association of p300 with chromatin and its ability to acetylate histones. K9 methylation did not inhibit the association of p300 with chromatin [p300 versus p300 and G9a(SET), $P = 0.19$]; however, it did block p300-induced H3 acetylation. H3 dimeK9, H3 dimethyl-K9; acH3, acetyl-H3.

histone H4 acetylation (Fig. 4B, compare the middle lane with the leftmost lane for H4 acK5 and H4 acK16). These data indicate that K9 methylation prevents H3 acetylation by p300 but does not inhibit the general association of p300 with chromatin.

SUV39H1 suppresses transcription and recruits HP1 to chromatin through multiple mechanisms. Next we wished to understand why SUV39H1 but not G9a(SET) was capable of recruiting HP1 to chromatin, whereas both led to H3-K9 methylation. Toward this end, we first wished to determine whether the HMT activity of SUV39H1 is required for its ability to recruit HP1 to chromatin. For this purpose, we made several additional Gal4(DBD)-SUV39H1 fusion constructs (Fig. 5A) including a full-length H324K mutant that abolishes enzymatic activity (22, 40), the C-terminal SET domain with or without a H324K mutation, and the N-terminal region that contains the previously identified HP1 interaction domain (the first 39 amino acids) (61) and the CD (SUVΔC). The mRNAs derived from these constructs were injected into *Xenopus* oocytes, and

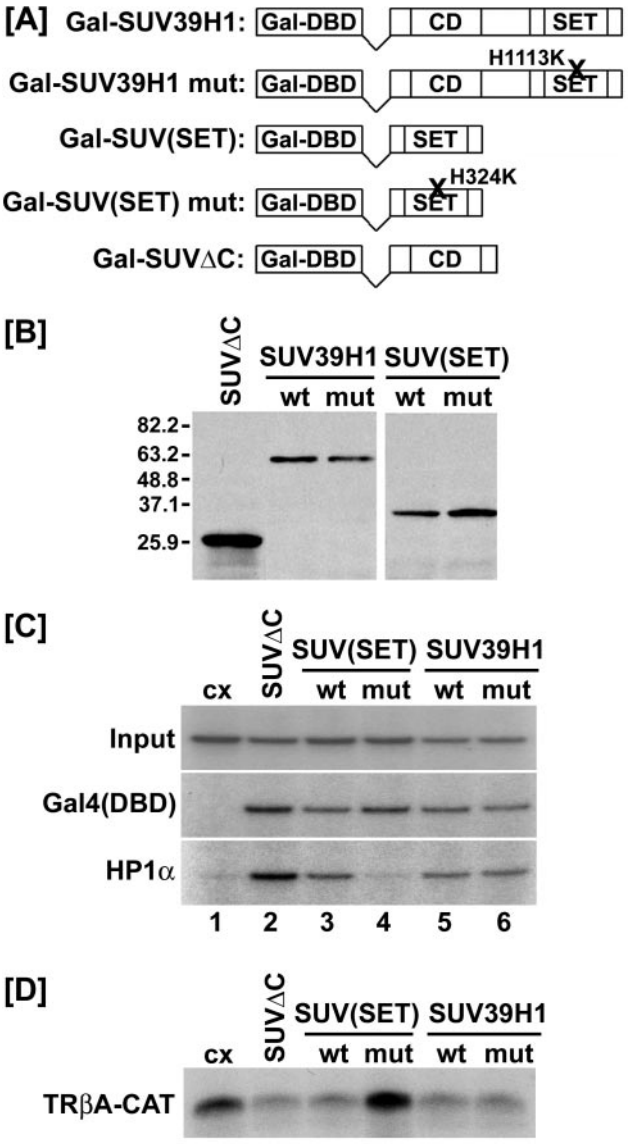

FIG. 5. SUV39H1 suppresses transcription and recruits HP1 through multiple mechanisms. (A) Schematic diagrams of the various Gal-SUV39H1 fusion proteins. mut, mutant. (B) Expression of the various Gal-SUV39H1 fusion proteins in *Xenopus* oocytes the day after injection of their corresponding in vitro-synthesized mRNA. Western blots were performed using a Gal4(DBD)-specific antibody. The positions of molecular mass markers (in kilodaltons) are shown to the left of the blots. wt, wild type; mut, mutant. (C) Recruitment of HP1 to chromatin by the Gal-SUV39H1 fusion proteins. Groups of oocytes were injected with mRNAs encoding the various Gal-SUV39H1 fusion proteins as indicated, and ChIP assays were performed using anti-Gal4(DBD) or anti-HA tag antibody to determine binding of Gal4 fusions or HP1α to chromatin, respectively. Note that for the full-length protein, the recruitment of HP1α is not dependent on HMT activity, whereas the recruitment of HP1α by SUV(SET) is dependent on HMT activity. cx, no mRNA injected. (D) Effects of the Gal-SUV39H1 fusion proteins on transcription of the 4xUAS-TRβA-CAT reporter gene as determined by primer extension. Note that for the full-length protein, repression is not dependent on the HMT activity, whereas repression by SUV(SET) is HMT activity dependent.

expression of the expected protein products was confirmed by Western blotting (Fig. 5B).

To determine the ability of these various SUV39H1 derivatives to recruit HP1, we injected groups of *Xenopus* oocytes with mRNA encoding HA-HP1α together with the SUV39H1 derivatives as indicated, followed by injection of the 4xUAS-TRβA-CAT reporter using the experimental design illustrated in Fig. 1A. ChIP assays were then performed using anti-HA tag antibodies to determine whether HA-HP1α was recruited to chromatin. Anti-Gal4(DBD) antibodies were used in ChIP assays to determine whether the Gal4 fusion proteins bound the reporter DNA (Fig. 5C). We found that for the full-length protein, the recruitment of HP1 was independent of SUV39H1's HMT activity (Fig. 5C, compare lanes 6 and 5). However, in the context of the SET domain alone, the HMT activity of SUV39H1 was essential for recruitment (Fig. 5C, compare lanes 4 and 3). Such discrepancy can be explained by the observation that the N-terminal domain (SUVΔC) alone was sufficient to recruit HP1, consistent with the presence of a HP1 interaction domain in the N terminus. These data indicate that two different regions in SUV39H1 can recruit HP1 to chromatin. The first involves the N-terminal region, which is known to associate with HP1 and histone deacetylase activity (7, 43, 53, 61). The second involves the SET domain and requires its K9 HMT activity.

Primer extension assays showed that the N-terminal (SUVΔC), SET-containing, and full-length Gal4 fusion proteins could repress transcription of the chromatin-assembled 4xUAS-TRβA-CAT reporter. With the full-length protein, the HMT activity of SUV39H1 was not required for repression. However, in the absence of the N-terminal region that contains the CD, the HMT activity of SUV39H1 was required to suppress transcription of the reporter gene (Fig. 5D).

The degree of K9 methylation is not a factor in determining whether HP1 will be recruited to chromatin. The above results raise the question as to why the SUV39H1 SET domain, but not the G9a SET domain, is able to induce HP1 recruitment to chromatin. One possible explanation is that the two HMTs induce different degrees of K9 methylation. Lysines can be mono-, di-, or trimethylated. From in vitro binding experiments, it is known that HP1 binds a bit more strongly to trimethyl-K9 than to dimethyl-K9 (12) and that SUV39H1 preferentially confers trimethylation to K9 (38, 41). Therefore, we examined whether there is a difference in the methylation state induced by G9a and SUV39H1 and whether this difference could account for the differences in HP1 recruitment. *Xenopus* oocytes were injected with mRNA encoding either Gal-G9a(SET), Gal-SUV39H1, or Gal-SUV(SET) and the 4xUAS-TRβA-CAT reporter. Then, ChIP assays were performed using antibodies raised against the Gal4(DBD), dimethyl-K9, or trimethyl-K9.

As shown in Fig. 6A, all three Gal4 fusion proteins bound the reporter DNA. Gal-G9a(SET) induced both dimethylation and trimethylation of lysine 9 (Cx versus G9a, $P < 0.001$), whereas the full-length Gal-SUV39H1 induced only trimethylation (Cx versus SUV39H1; dimethyl-K9, $P = 0.21$; trimethyl-K9, $P < 0.001$). Interestingly, the SET domain of SUV39H1 induced H3-K9 dimethylation, but not trimethylation [Cx versus SUV(SET); dimethyl-K9, $P = 0.002$; trimethyl-K9, $P = 0.38$]. These data indicate that the region of SUV39H1 N terminal to the SET domain may regulate the HMT activity of its SET domain in a manner reminiscent of the effect of mAM on ESET/SETDB1 HMT activity (55). Nevertheless, these data also indicate that the methylation status of lysine 9 is probably not a major factor in determining why the SET domain of SUV39H1, but not the SET domain of G9a, is capable of recruiting HP1 to chromatin. Gal-G9a(SET) cannot recruit HP1 even though it strongly induces both di- and trimethylation of K9, whereas both the entire length and the SET domain alone of SUV39H1 can recruit HP1, even though one induces mainly trimethylation and the other induces mainly dimethylation.

Identification of a novel HP1 interaction domain within the SUV39H1 SET domain. Another possible explanation for why the SUV39H1 SET domain can recruit HP1 but the G9a SET domain cannot is that the SUV39H1 SET domain, but not the G9a SET domain, directly interacts with HP1. To test this hypothesis, GST pull-down experiments were performed using GST-HP1α and in vitro-translated Gal-G9a(SET), Gal-SUV39H1, Gal-SUVΔC, or Gal-SUV(SET). The full-length and ΔC SUV39H1 constructs were used as positive controls, because the extreme N terminus of SUV39H1 possesses a known HP1 interaction site (43, 61). The results in Fig. 6B showed that the SET domain of G9a did not interact with HP1α directly, whereas the SUV39H1 SET domain bound to HP1α well. Interestingly, the Gal-SUV(SET) H324K mutant retained the in vitro HP1-binding activity, even though it does not recruit HP1 to chromatin in vivo. Thus, unlike the G9a SET domain, there is a novel HP1 interaction site within the SUV39H1 SET domain.

Since the C-terminal HP1 interaction site in SUV39H1 has not been previously reported, we further mapped the interaction site. Four truncations of the SUV39H1 C terminus were in vitro translated and tested for their ability to bind GST-HP1α. The HP1 interaction site was found to lie within the C-terminal half of the SET domain excluding the post-SET region (Fig. 6C). These data indicate that SUV39H1 possesses two HP1 interaction domains, one in the N terminus and one in the C-terminal SET domain.

We next tested whether the newly identified C-terminal HP1 interaction site could interact with HP1 in a coimmunoprecipitation experiment. HA-HP1α was coexpressed with Gal-G9a(SET) (negative control), Gal-SUV39H1, Gal-SUVΔC, or Gal-SUV(SET) in *Xenopus* oocytes. Coimmunoprecipitations were performed using either anti-HA or anti-Gal4(DBD) antibody for IP and the complementary antibody for Western blotting. As shown in Fig. 6D, although coimmunoprecipitation of full-length SUV39H1 and SUVΔC with HP1α were readily detected, no significant coimmunoprecipitation was observed between SUV(SET) and HP1α. Therefore, the direct interaction between HP1α and the SUV39H1 N terminus is likely much weaker that the interaction with the C terminus and could not be detected by coimmunoprecipitation under our experimental conditions.

A two-interaction model for recruitment of HP1 to chromatin. The above results suggest that the failure for G9a SET to recruit HP1 is likely due to a lack of direct protein-protein interaction between G9a SET and HP1. Furthermore, the above results show that the SUV39H1 SET domain mutant maintains a direct interaction with HP1 but does not recruit

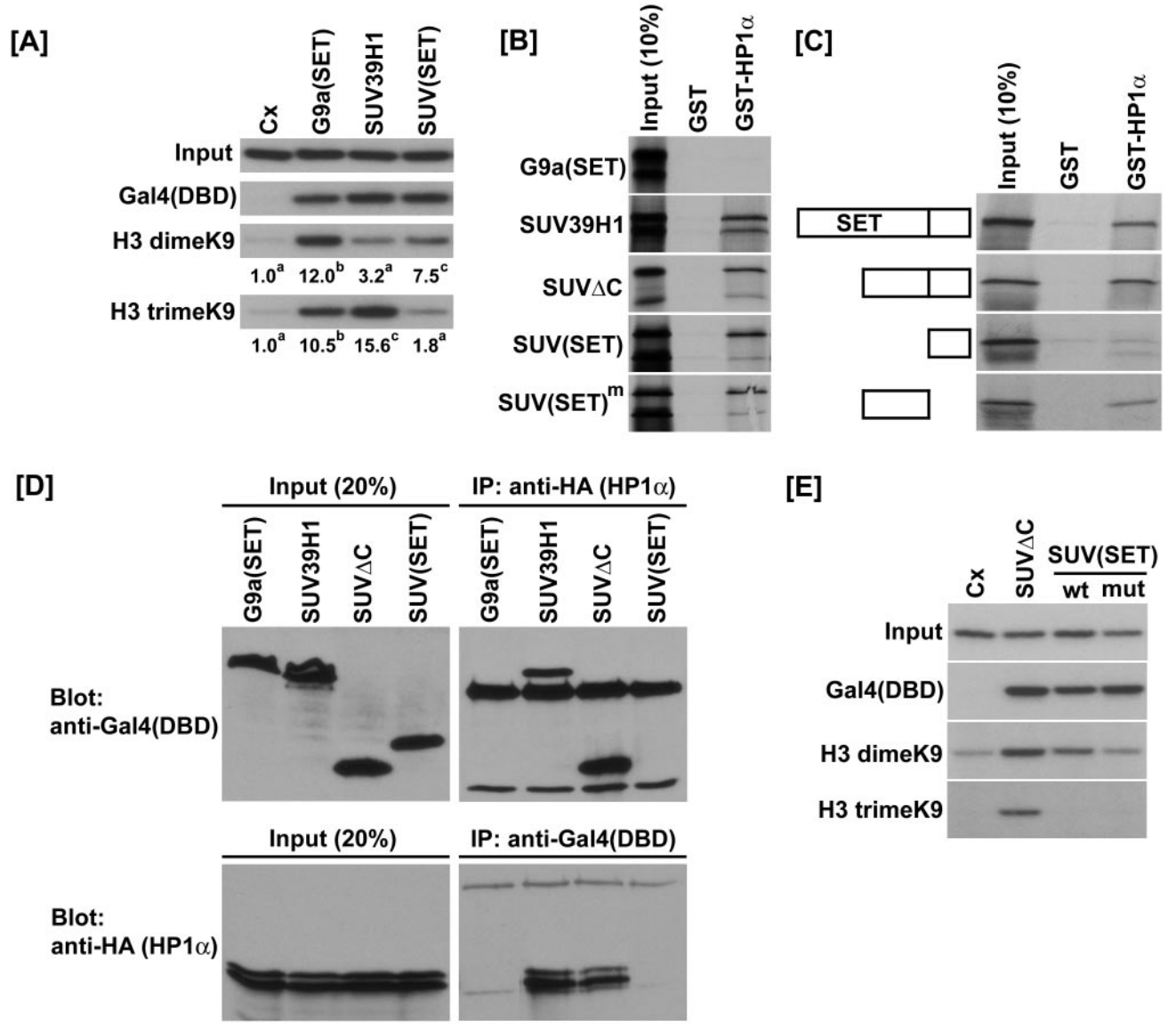

FIG. 6. Both lysine 9 methylation and a HP1-interacting protein must be targeted to chromatin for HP1 recruitment. (A) ChIP assay to compare di- and trimethylation of K9 induced by Gal-G9a(SET), Gal-SUV39H1, and Gal-SUV(SET). The fold difference between the LS mean for each group and the control group (Cx) is reported beneath each blot. Note that SUV(SET) mainly induced K9 dimethylation, while the full-length SUV39H1 primarily induced trimethylation of K9. H3 dimeK9, H3 trimeK9, H3 dimethyl-K9; H3 trimethyl-K9. (B) Each construct was tested for its ability to directly interact with HP1α by an in vitro GST pull-down assay. For a control, an equal amount of GST protein was used. SUV(SET)^m, mutant SUV(SET). (C) The novel HP1 interaction site in the C terminus of SUV39H1 was further mapped to the C-terminal half of the SET domain excluding the post-SET region by in vitro pull-down assay. (D) Reciprocal coimmunoprecipitations to study the two HP1 interaction sites on SUV39H1. HA-HP1α easily coimmunoprecipitated with the N terminus of SUV39H1; however, the interaction between HP1α and the SUV39H1 SET domain was not detected, implying a weak protein-protein interaction. (E) Tethering the N-terminal region of SUV39H1 (SUVΔC) to chromatin resulted in increased H3-K9 methylation as revealed by ChIP assay. Cx, no mRNA injected; wt, wild type; mut, mutant.

HP1 to chromatin in vivo. Together, these results suggest that stable HP1 recruitment to chromatin requires two conditions: the presence of methyl-K9 and a direct protein-protein interaction with SUV39H1. If this hypothesis were true, we would expect that the SUV39H1 N-terminal fragment (SUVΔC), which was able to recruit HP1 to chromatin in vivo (Fig. 5C), must also induce K9 methylation. To test this idea, *Xenopus* oocytes were injected with mRNA encoding Gal-SUVΔC. Other groups of oocytes were injected with mRNA encoding Gal4 fusions of the SUV39H1 SET domain or SET H324K mutant as positive and negative controls, respectively. In support of our hypothesis, the SUV39H1 N terminus alone was sufficient to induce di- and trimethylation of K9 (Fig. 6E). The wild-type, but not mutant, SUV39H1 SET domain induced only K9 dimethylation. Since the SUV39H1 N terminus alone lacks intrinsic H3-K9 enzymatic activity (data not shown), the observed K9 methylation is likely a result of dimerization with endogenous SUV39H1 or association with a HMT-containing

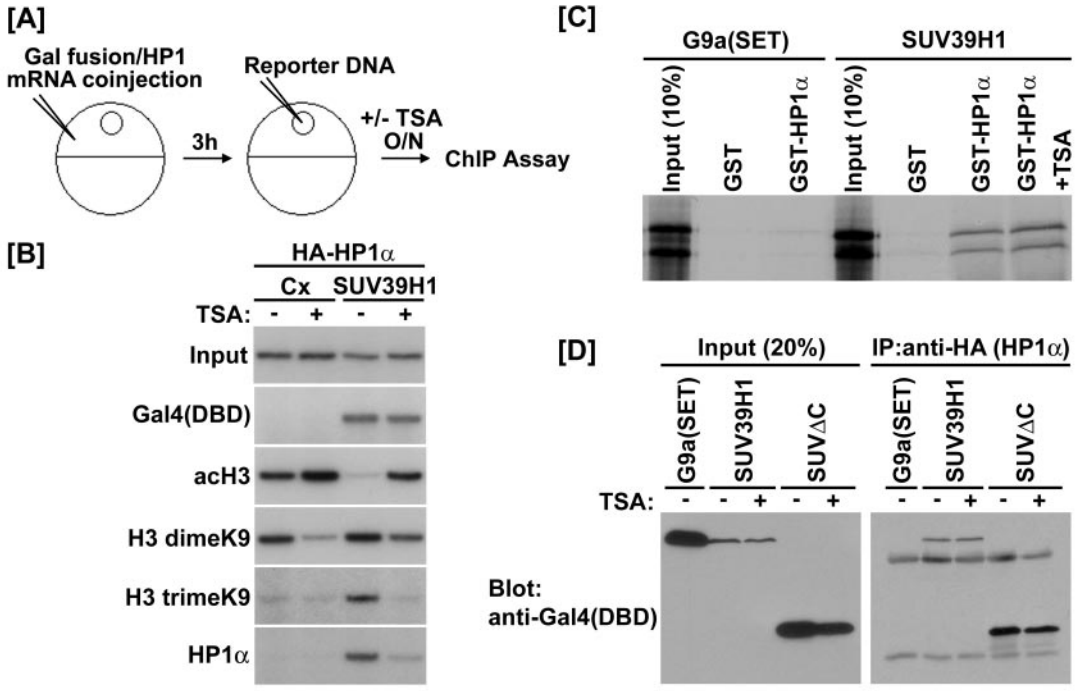

FIG. 7. Targeting a HP1-interacting protein to chromatin is not sufficient for HP1 recruitment in the absence of lysine 9 methylation. (A) Experimental design. Oocytes were divided into four groups. All groups received mRNA encoding HA-HP1α. Two groups received an additional injection of Gal-SUV39H1 mRNA, while the other two groups did not (Cx groups). After 3 h, all oocytes were injected with the 4xUAS-TRβA-CAT reporter and then immediately treated with TSA (1.65 μM) or not treated with TSA. After overnight (O/N) incubation, the oocytes were harvested for ChIP analysis using the indicated antibodies. (B) In the absence of TSA (−), Gal-SUV39H1 methylated K9 and recruited HP1 to chromatin; however, in the presence of TSA (+), K9 methylation was reduced and HP1 recruitment was impaired. acH3, acetyl-H3; H3 dimeK9, H3 dimethyl-K9; H3 trimeK9, H3 trimethyl-K9. (C) TSA did not affect the in vitro binding of SUV39H1 to HP1α as determined by GST pull-down assay. (D) TSA did not affect the in vivo binding of SUV39H1 to HP1α as determined by coimmunoprecipitation.

complex. Indeed, it was recently reported that the N terminus of *Drosophila* SU(VAR)3-9 mediates its dimerization (9). These data support the hypothesis that both K9 methylation and a HP1-interacting protein must be targeted to chromatin for HP1 recruitment.

Targeting a HP1-interacting protein to chromatin is not sufficient for HP1 recruitment in the absence of lysine 9 methylation. To further test this hypothesis, we determined the ability of SUV39H1 to recruit HP1 to chromatin in the absence of methyl-K9. To do this, we took advantage of the ability of TSA to block H3-K9 methylation as a result of its effect on histone acetylation. *Xenopus* oocytes were injected with mRNA encoding Gal-SUV39H1 and HP1α and the 4xUAS-TRβA-CAT reporter. Oocytes were then treated with or without TSA overnight (Fig. 7A). ChIP assays were performed to test HP1 recruitment and K9 methylation under this condition (Fig. 7B). TSA did not affect the binding of Gal-SUV39H1 to the reporter; however, it did increase acetylation of H3 and block K9 methylation. Importantly, TSA treatment severely impaired the recruitment of HP1 to chromatin (Fig. 7B). This result argues for an essential role for H3-K9 methylation in the recruitment of HP1 to chromatin by SUV39H1.

To exclude the possibility that TSA may affect the direct binding of HP1 to SUV39H1, we performed a GST pull-down assay in which we tested the binding of in vitro-translated Gal-SUV39H1 to GST-HP1α in the presence or absence of TSA (Fig. 7C). In vitro-translated Gal-G9a(SET) was used as a negative control for the assay. Indeed, TSA did not affect the in vitro interaction of HP1α with SUV39H1. In addition, we performed coimmunoprecipitations to examine whether TSA inhibited the interaction between HP1 and SUV39H1 in *Xenopus* oocytes. The results in Fig. 7D show that the full-length and SUVΔC constructs immunoprecipitated with HA-HP1α and TSA treatment did not affect this interaction. These data, together with the ChIP result that TSA did not affect the binding of Gal-SUV39H1 to chromatin, demonstrate that although SUV39H1 is bound to chromatin and presumably can still directly interact with HP1 in solution, it fails to recruit HP1 to chromatin in the absence of methyl-K9. This observation supports our model that both K9 methylation and a direct interaction between SUV39H1 and HP1 are required for the chromatin targeting of HP1.

DISCUSSION

According to the histone code theory, there is an abundance of proteins that specifically recognize and bind to histones when they are posttranslationally modified in a specific way. HP1 is one such protein, which has repeatedly been shown to bind to the H3 N-terminal tail when it is methylated on lysine 9 (4, 12, 13, 20, 29, 32). It is thought that methyl-K9 facilitates formation of heterochromatin and represses transcription by recruiting HP1, which interacts with itself and other factors to create a compact chromatin structure that is not permissive to

transcription. In this study, we have shown that lysine 9 methylation alone is not sufficient to recruit HP1 to chromatin; however, it is sufficient to suppress transcription. It represses transcription in the absence of HP1 through a mechanism involving histone deacetylation. We performed several experiments to determine why SUV39H1, but not G9a, is able to recruit HP1 to chromatin and conclude that, in addition to methyl-K9 binding, stable HP1 recruitment requires a secondary direct protein-protein interaction.

K9 methylation and transcriptional repression. In this study, we showed that tethering the HMT domain of G9a to chromatin caused H3-K9 methylation but failed to recruit HP1. Furthermore, we showed that tethering the wild-type protein, but not the inactive-HMT H1113K mutant, led to robust transcriptional repression of all three reporters we tested. Although Tachibana and colleagues (49) reported that G9a has weak HMT activity toward lysine 27 of H3, we were unable to detect K27 methylation by ChIP assays using two different methyl-K27 antibodies, including one from Upstate Biotechnology, which very effectively immunoprecipitates methyl-H3-K27 from HeLa cell extracts in our laboratory (H. G. Yoon and J. Wong, unpublished observations). In support of our observation, a recent report comparing wild-type and *G9a*-null ES cells concluded that G9a does not regulate K27 methylation in mammalian chromatin (38). Altogether, we presume that our Gal-G9a(SET) protein does not methylate K27 or does so to an extent below the detection limit of our ChIP experiments. Therefore, we conclude that repression by tethering G9a(SET) to chromatin is most likely the direct or indirect consequence of H3-K9 methylation, although the possibility that G9a(SET) also methylates other proteins involved in transcription cannot be excluded.

A previous characterization of the G9a HMT reported that when fused to Gal4(DBD), the HMT domain of G9a was able to suppress transcription of a transfected luciferase reporter gene (50). However, in their experiments, the repression by G9a was insensitive to TSA treatment. In contrast, we report here that repression by G9a is dependent on histone deacetylation and can be completely blocked by TSA, illustrating a central role for histone deacetylation in mediating the transcriptional repression induced by K9 methylation. Our finding that repression by G9a is sensitive to TSA is likely due to the nature of our reporter system. In the *Xenopus* oocyte, our reporter gene is assembled into chromatin via a replication-coupled pathway (2). In transient-transfection experiments, the reporter gene does not adopt a true chromatin structure (16). A previous study showed that targeting either the SET domain of G9a or SUV39H1 to the promoter of the endogenous *VEGF-A* gene repressed its transcription and concluded that H3-K9 methylation causes repression (45); however, how K9 methylation caused transcriptional repression was not clearly demonstrated in that study.

Our experiments show that histone deacetylation is required for the repressive function of methyl-K9. Specifically, we found that K9 methylation induces deacetylation of both H3 and H4. This effect on histone acetylation is probably not a result of direct association of HDACs with the G9a SET domain due to the following reasons. First, by immunoprecipitation and in vitro HDAC assays, we have not observed any significant HDAC activity associated with G9a (J. Li and J. Wong, unpublished results). Second, by using immobilized GST-G9a(SET) proteins, we failed to pull down HDAC activity above the background level from HeLa nuclear extracts or *Xenopus* oocyte extracts (data not shown). We also failed to detect the recruitment of endogenous *Xenopus* RPD3 (HDAC1/2) or expressed Flag-HDAC1 or Flag-HDAC3 by Gal-G9a(SET) in ChIP assays (data not shown). Finally, as histone deacetylation was not observed when the Gal-G9a(SET) H1113K mutant was used, the observed deacetylation is most likely a consequence of K9 methylation.

K9 methylation could affect histone deacetylation by facilitating the recruitment and/or activity of HDACs or by preventing the association and/or inhibiting the activity of histone acetyltransferases with chromatin or both. Because most histone acetyltransferases, such as P/CAF, Gcn5, and CBP/p300, possess bromo domains that bind preferentially to acetylated lysine (8, 36), we attempted to determine whether H3-K9 methylation affects the association of the p300 acetyltransferase with chromatin. We found that K9 methylation does not inhibit the association of p300 with chromatin but is able to prevent p300-induced H3 acetylation. These data suggest that methyl-K9 does not prevent the association of p300 with chromatin but may block its HAT activity, in agreement with a previous in vitro study showing that H3-K9 methylation inhibits histone acetylation (56). Although the binding of p300 to chromatin was not affected by H3-K9 methylation, it remains to be tested whether the chromatin association of other HATs, such as p/CAF and Gcn5, is affected. Furthermore, our data cannot exclude the possibility that methyl-K9 also promotes histone deacetylation. Although there is no data in the literature to suggest that HDAC complexes bind specifically to methylated H3-K9 tails, the HDAC1/2-containing nucleosome remodeling and deacetylase (NuRD) complex has been shown to bind preferentially to the unmodified H3 tail (34, 64). Given the antagonizing effect of K9 methylation on acetylation, one can envision that K9 methylation could inhibit histone acetylation and thus facilitate binding and subsequent further deacetylation of histones by NuRD. The combined effect would presumably lead to deacetylation of both H3 and H4.

A two-interaction model for the relationship between K9 methylation and HP1 recruitment. Although methyl-K9 serves as a binding site for HP1 in vitro and has been shown to be important for appropriate heterochromatin association of HP1 in vivo (4, 13, 20, 29, 39), we find that K9 methylation itself is necessary but not sufficient for targeting HP1 to chromatin. First, the SUV39H1 SET mutant maintains its protein-protein interaction with HP1 in vitro but is unable to target HP1 to chromatin in vivo (Fig. 5C and 6B). Second, SUV39H1 is unable to recruit HP1 to chromatin if K9 methylation is indirectly blocked by TSA treatment (Fig. 7B). Third, G9a induced both di- and trimethylation of K9 but failed to recruit HP1 to chromatin (Fig. 1D). Together, our data support a working model (Fig. 8) that in vivo HP1 cannot independently "read" the histone modification coded in methyl-K9 and that the HP1-methyl-K9 interaction functions in the context of other protein-protein interactions, such as interactions with SUV39H1, which provide an additional stabilizing force essential for targeting HP1 to chromatin. This direct protein-protein interaction with HP1 could be provided by either one of the two HP1 interaction sites on SUV39H1, one in its N terminus that was

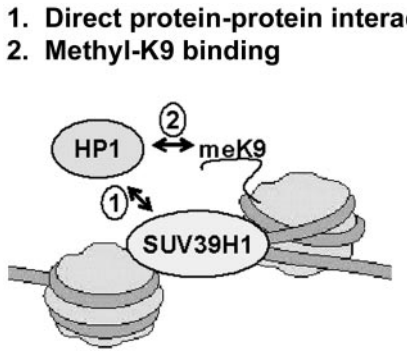

FIG. 8. Two-interaction model for HP1 recruitment to chromatin. Based on the data presented here and the observations of others (see Discussion), K9 methylation by itself is insufficient to recruit HP1 to chromatin. Stable association of HP1 with chromatin involves multiple interactions. HP1 recruitment to chromatin by SUV39H1 requires both a direct interaction between HP1 and SUV39H1 and methyl-K9 (meK9) binding.

previously described to interact with the chromo shadow domain of HP1 (43, 61) and one in its SET domain as identified in this study (Fig. 6B and C).

The *Drosophila Su(var)3-9* gene is one of a few antipodal modifiers of position effect variegation (PEV) exhibiting haplo-suppressor and triple-enhancer effects (52, 59). The few proteins that display this dose-dependent effect on PEV were generally accepted to be building blocks of heterochromatin, because increased expression of the protein caused spreading of heterochromatin but did not nucleate new heterochromatic sites (24). The data presented in this study support the idea that SUV39H1/SU(VAR)3-9 is a structural component of heterochromatin, because its direct protein-protein interaction with HP1 was required for stable association of HP1 with chromatin.

In mammalian cells, trimethyl-H3-K9 is enriched in pericentric heterochromatin, whereas mono- and dimethyl-H3-K9 are diffusely scattered throughout silent euchromatin (41). HP1 (α and β) and SUV39H1 colocalize mainly to pericentric heterochromatin (1, 31). A similar pattern of colocalization has also been reported in *Drosophila* polytene chromosome spreads (6, 14, 23). Since HP1 and SUV39H1 concentrate to pericentric heterochromatin, much of the methyl-K9 in euchromatic regions is not associated with HP1 (6), supporting the idea that K9 methylation alone is insufficient to recruit HP1 to chromatin. Our data provide a molecular explanation for this observation by illustrating that, in addition to K9 methylation, a protein-protein interaction between HP1 and SUV39H1 is also essential for HP1 recruitment. In addition, our data also argue against the possibility that trimethyl-K9 is the determinant for pericentric heterochromatin localization of HP1, since the SUV39H1 SET domain by itself is capable of recruiting HP1 but mainly gives rise to dimethyl-K9, whereas G9a(SET) directs both di- and trimethylation of K9 but fails to recruit HP1.

It should be emphasized here that the association of HP1 with heterochromatin is likely a complex event involving not only the interaction between HP1 and methyl-K9 and the interaction between HP1 and SUV39H1 as shown here. For instance, it was reported that the stable association of HP1 with heterochromatin also requires a RNA component(s) (25, 28). Consistent with this idea, heterochromatin silencing and HP1 localization are also dependent on RNA interference (RNAi) machinery both in *Drosophila* and fission yeast (37, 54). In addition, the hinge region of HP1 has intrinsic DNA- and chromatin-binding activity in vitro (26). Finally, a recent study showed that the stable Triton X-100-resistant association of HP1 with heterochromatin also requires the interaction of the HP1 chromo shadow domain with PXVXL motif-containing proteins (51). Thus, stable association of HP1 with heterochromatin is likely a multistep process involving interactions with SUV39H1, binding of methyl H3-K9, further stabilization by RNA components, and other protein-protein interactions.

Another interesting observation in this study was the difference in the degree of lysine 9 methylation induced by the full-length SUV39H1 protein and the SUV39H1 SET domain protein. Full-length SUV39H1 induced mainly trimethylation of K9, whereas the SUV39H1 SET domain induced mainly dimethylation. Our result that tethering full-length SUV39H1 to chromatin gave rise to mainly H3-K9 trimethylation is consistent with two recent studies showing that in mouse embryonic fibroblast cells Suv39h1 and Suv39h1 are mainly responsible for H3-K9 trimethylation in pericentric heterochromatin (38, 41). We propose that the N-terminal half of SUV39H1 possesses a property that modifies the enzymatic activity of the SET domain. In support of this idea, it was recently reported that the N terminus of SU(VAR)3-9 enhances the HMT activity of its C-terminal SET domain (9), although it was not shown whether the N terminus also affects the degree of methylation induced by the SET domain. The trimethylation activity of the full-length SUV39H1 could also be the result of another factor that associates with the SUV39H1 and modifies the activity of the enzyme. There is precedence for this idea, because the mAM protein tightly associates with the K9-specific HMT ESET/SETDB1 and converts its enzymatic activity from di- to trimethylation (55).

In conclusion, the data presented here illustrate that stable recruitment of HP1 to chromatin requires two interactions (Fig. 8). The first is the well-known interaction between HP1 and methyl-K9, and the second is a direct protein-protein interaction. These results illustrate that the histone code is interpreted in the context of other chromatin-associated factors and it is the combination of interactions with histone modifications and other factors that determines whether a protein will be recruited to chromatin.

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